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Transcription Factor Tfec Contributes to the IL-4-Inducible Expression of a Small Group of Genes in Mouse Macrophages Including the Granulocyte Colony-Stimulating Factor Receptor¹

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Expression of the mouse transcription factor EC (Tfec) is restricted to the myeloid compartment, suggesting a function for Tfec in the development or function of these cells. However, mice lacking Tfec develop normally, indicating a redundant role for Tfec in myeloid cell development. We now report that Tfec is specifically induced in bone marrow-derived macrophages upon stimulation with the Th2 cytokines, IL-4 and IL-13, or LPS. LPS induced a rapid and transient up-regulation of Tfec mRNA expression and promoter activity, which was dependent on a functional NF- κ B site. IL-4, however, induced a rapid, but long-lasting, increase in Tfec mRNA, which, in contrast to LPS stimulation, also resulted in detectable levels of Tfec protein. IL-4-induced transcription of Tfec was absent in macrophages lacking Stat6, and its promoter depended on two functional Stat6-binding sites. A global comparison of IL-4-induced genes in both wild-type and Tfec mutant macrophages revealed a surprisingly mild phenotype with only a few genes affected by Tfec deficiency. These included the G-CSFR (*Csf3r*) gene that was strongly up-regulated by IL-4 in wild-type macrophages and, to a lesser extent, in Tfec mutant macrophages. Our study also provides a general definition of the transcriptome in alternatively activated mouse macrophages and identifies a large number of novel genes characterizing this cell type. *The Journal of Immunology*, 2005, 174: 7111–7122.

The differentiation of macrophages from hemopoietic progenitor cells requires the regulated expression of several specific transcription factors that control lineage commitment, differentiation, and cell type-specific gene regulation. These include members of the Ets, C/EBP, corticosterone-binding factor families and several other transcription factors (1, 2). In particular, transcription factors such as PU.1 that show a restricted or cell type-specific expression pattern seem to play a major role in regulating macrophage differentiation (1–3). We previously showed that the expression of mouse transcription factor EC (Tfec)⁴ mRNA is also restricted to cells of the monocyte-macrophage lineage (4).

Tfec, a member of the microphthalmia-TFE (MiT) subfamily of basic helix-loop-helix (bHLH) transcription factors, was originally

identified in a rat chondrosarcoma (2, 5). Although human TFEC exists as multiple transcript variants resulting from alternative promoter usage or alternative splicing that either contain or lack an N-terminal activation domain (6, 7), rodents apparently express a single isoform of Tfec without the N-terminal activation domain. Early data suggested that Tfec forms homodimers or heterodimers with other MiT proteins and acts as a transcriptional inhibitor (5). More recent work indicates that both human and mouse Tfec contain a C-terminal activation domain, and that the ability of Tfec to repress or activate a reporter gene in transient cotransfection assays depends on both the promoter context and the cell type used (6, 8, 9). The restricted expression of the mouse transcription factor Tfec is controlled by a typical TATA-less, myeloid-type promoter that contains several Ets motifs that bind the myeloid- and B cell-restricted transcription factor PU.1 (4). Although some macrophage-related defect was anticipated based upon the expression pattern, mice lacking Tfec are indistinguishable from their wild-type littermates; they are viable and fertile, normally pigmented, have normal eyes and mast cells, and show no osteopetrosis (10). One possible explanation for the lack of a phenotype is that Tfec in macrophages is coexpressed with the other known MiT members (4) and is genuinely redundant. Alternatively, the penetrance of mutations could be influenced by genetic background. For example, the penetrance of mutations in both the related microphthalmia transcription factor and PU.1 is strongly influenced by background genotype (11).

A third possibility is that Tfec has a nonredundant function in some state of macrophage activation that is only required in response to a particular challenge. Macrophages detect and respond to a plethora of physiological and pathophysiological cues, including microbial agents and a large number of chemo- and cytokines,

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⁴ Abbreviations used in this paper: Tfec, transcription factor EC; bHLH, basic helix-loop-helix; BMDC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; BMMC, bone marrow-derived mast cell; MiT, microphthalmia-Tfe; msh, muscle segment homeobox; SCF, stem cell factor.

with profound functional changes (12). The activation program of mononuclear phagocytes, e.g., at sites of inflammation, is mainly influenced by the local cytokine milieu. Although Th1 cytokines and microbial agents induce classically activated macrophages that produce proinflammatory mediators and effectively kill microorganisms, Th2 cytokines, e.g., IL-4, support a distinct activation program leading to alternatively activated macrophages that tune inflammatory responses, promote tissue repair, and scavenge debris (13). To gain insight into a possible regulatory role of Tfec in activated macrophages under pathophysiological conditions, e.g., inflammation or cancer, we studied the expression of Tfec in macrophages treated with different pro- and anti-inflammatory mediators. Most strikingly, the Th2 cytokine IL-4 strongly induced Tfec mRNA and protein in a Stat6-dependent fashion, implicating Tfec as a secondary mediator of IL-4 signals in alternatively activated macrophages. Using cDNA microarray analysis, we were able to identify a small group of genes that are affected by Tfec deficiency in mouse macrophages, including the G-CSFR gene (*Csf3r*) whose up-regulation in IL-4-induced, alternatively activated macrophages was at least partially Tfec dependent. Our analysis also provides a global view of genes that shape the phenotype and function of alternatively activated macrophages. In addition to Tfec, we identify a number of novel transcription factors, including muscle segment homeobox (*msh*)-like homeobox gene 3 (*Msx3*), early growth response 2 (*Egr2*, Krox20), bHLH transcription factor STRA13/Dec1 (*Bhlhb2*), IL-3-induced NF (*Nfil3*, E4bp4), Krueppel-like factor 4 (*Klf4*, Gklf), basic transcription element-binding protein 1 (*Bteb1*), and *ets* variant gene 3 (*Etv3*, PE1/Mets), that are significantly induced by IL-4 and may act as secondary mediators of the IL-4 response in mouse macrophages.

Materials and Methods

Chemicals

All chemical reagents used were purchased from Sigma-Aldrich unless otherwise noted. Protease inhibitors were obtained from Roche. Oligonucleotides were synthesized by TIB Molbiol. Antisera for supershift analyses were purchased from Santa Cruz Biotechnology. Recombinant mouse cytokines were purchased from PeproTech.

Cells

The cell line RAW264.7 (American Type Culture Collection) was cultivated in RPMI 1640 medium (BioWhittaker) and 10% FCS. To obtain bone marrow-derived macrophages (BMM), bone marrow-derived dendritic cells (BMDC), or bone marrow-derived mast cells (BMMC), bone marrow was extracted from the femurs of 8- to 12-wk-old mice. Cells from several animals were pooled before plating into noncharged bacteriological plates at 5×10^5 cells/ml. To obtain BMM, the cells were cultured in RPMI 1640, 10% FCS, and 200 ng/ml human rCSF-1 (Cetus) for 5 days. On day 5, the medium was replaced, and cells were harvested on day 6 and replated at a density of 10×10^6 cells/10-cm tissue culture dish at 5×10^5 cells/ml, with CSF-1 added back overnight. Experimental time courses were started on day 7. BMDC were obtained and cultured using mouse rGM-CSF (10 ng/ml; PeproTech) as described by Lutz et al. (14). To obtain BMMC, bone marrow cells were cultured in RPMI 1640 and 10% FCS supplemented with stem cell factor (SCF; 50 ng/ml; PeproTech) and IL-4 (40 ng/ml; PeproTech; treatment A) or with SCF (50 ng/ml; PeproTech) and IL-3 (5 ng/ml; PeproTech; treatment B). Medium and cytokines were replaced every 3 days. Cells were harvested after 2 wk of culture.

Mice

BALB/c mice were obtained from Charles River Laboratories. The mutant mouse strain deficient in Tfec (129SvJ background) was generated by gene targeting, as described previously (10).

Flow cytometry

BMM were harvested and resuspended in PBS containing 10% FCS (Invitrogen Life Technologies). FcRs were blocked using anti-CD16/32 (clone 2.4G2; BD Pharmingen) on ice for 30 min. Cells were stained with PE-conjugated mAbs recognizing CD11b (Mac-1), CD115, CD204, F4/80

(all from Serotec), Ly-6G (Gr-1; BD Pharmingen), or isotype controls (Serotec and BD Pharmingen) in the dark for 45 min on ice. After staining, cells were washed twice with PBS containing 10% FCS. Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences).

RNA preparation and Northern blot analysis

Total RNA was isolated from different cell lines by the guanidine thiocyanate/acid phenol method. Electrophoresis, Northern blotting, and cDNA hybridization were conducted as described previously using a 1200-bp cDNA from Tfec coding region 4. Autoradiography was performed at -70°C , and bands were scanned with a Molecular Dynamics personal densitometer.

Microarray analysis and data handling

Two independent microarray experiments were performed using two different microarray platforms. Firstly, cDNA from 16-h IL-4-treated BMM (Tfec-deficient or wild type) were indirectly labeled with amino-alkyl-conjugated Cy3 (wild type) or Cy5 (Tfec^{-/-}), as described at (www.imb.uq.edu.au/groups/hume/), and hybridized at 65°C overnight to NIA 15K mouse cDNA microarrays (SRC, Microarray Facility). Slides were washed for 5 min in $2\times$ SSC/0.2% SDS buffer and scanned on a ScanArray 5000 confocal laser scanner. Molecularware (Digital Genome) was used to process the image, data were corrected for local background, and confidence status was flagged for empty spots, signal/noise ratio, spot ratio variability, and spot morphology. Data were imported into GeneSpring 4.2 (Silicon Genetics) for clustering and comparative analysis. Differentially expressed genes were identified as those elements that were induced or repressed >2 -fold from the wild-type condition and that were regulated by IL-4 in an independent microarray analysis comparing unstimulated and IL-4-stimulated wild-type BMM. A second experiment using RNA from 16-h IL-4-treated or untreated BMM (Tfec deficient or wild type) was performed using Affymetrix Mouse430A_2 arrays. Hybridization, cRNA labeling, and data handling were performed by Kompetenzzentrum für Fluoreszenz Bioanalytik.

Real-time PCR

Total RNA (2 μg) was reverse-transcribed using SuperScript II (Invitrogen Life Technologies). Real-time PCR was performed using the LightCycler (Roche) with the Quantitect kit (Qiagen) according to the manufacturer's instructions. The primers used are given in Table I. Cycling parameters were: denaturation at 95°C for 15 min and amplification at 95°C for 15 s, 57°C for 20 s, 72°C for 25 s for 55 cycles. The product size was initially controlled by agarose gel electrophoresis, and melting curves were analyzed to control for the specificity of the PCRs. The relative units were calculated from a standard curve with four different concentrations of log dilutions to the PCR cycle number at which the measured fluorescence intensity reaches a fixed value. Data were normalized against the housekeeping genes *Actb* (*Csf3r*) and *Hprt* (all others). The amplification efficiency (E) was calculated from the slope of the standard curve as: $E = 10^{-1/\text{slope}}$. The observed primer efficiencies are given in Table I. All primers were chosen to amplify a cDNA fragment that includes intron-exon borders in the corresponding genome sequence. The data from at least two independent analyses for each sample were averaged.

Plasmid construction and purification

Cloning of a 615-bp fragment of the TFEC proximal promoter has been described previously. Mutations of putative transcription factor binding sites were conducted by PCR-mediated mutagenesis using the following primers (mutated bases are underlined): NF κ B element, mTFEC-NF κ B_S (5'-CAG AGT CCA GTC GCT GCC CTA ATG G-3') and mTFEC-NF κ B_AS (5'-CCA TTA GGG CAG CGA CTG GAC TCT G-3'); outer STAT element, mTFEC-oS6M_S (5'-CTG TCT AAT CTT CTT GTC TAG ATC AAT TAT-3') and mTFEC-oS6M_AS (5'-CCA AGA ATA ATT GAT CTA GAC AAG AAG ATT-3'); middle STAT element, mTFEC-mS6M_S (5'-GGA CAG TGT TCT GAT CTA ACT TAC TTG TAA-3') and mTFEC-mS6M_AS (5'-TGC CTT ACA AGT AAG TTA GAT CAG AAC ACT-3'); and inner STAT element, mTFEC-iS6M_S (5'-CAT TGT AAA TTC TGG GCT CAA ATG TTT ATT-3') and mTFEC-iS6M_AS (5'-CCC CAT AAT AAA CAT TTG AGC CCA GAA TTT-3'). DNA sequence analysis was performed by GENEART. For transfections, plasmids were isolated and purified using the Endofree Plasmid Kit (Qiagen).

Transient and stable DNA transfections

For transient reporter analysis, RAW264.7 cells were transfected using SuperFect reagent (Qiagen) according to the manufacturer's instructions as

Table I. Oligonucleotide primers used for LightCycler real-time PCR

Gene	Primer Sequence (Sense and Antisense)	Efficiency (E)
<i>Arg1</i>	5'-GGA CTA GAT ATC ATG GAA GTG AAC CC-3' 5'-AGT CCC TGG CTT ATG GTT ACC C-3'	1.84
<i>Egr2</i>	5'-CCT TTG ACC AGA TGA ACG GAG TG-3' 5'-TGG TTT CTA GGT GCA GAG ATG GG-3'	1.98
<i>Klf4</i>	5'-CTG GCG AGT CTG ACA TGG C-3' 5'-CTC ACG CCA ACG GTT AGT CG-3'	1.94
<i>Etv3 (s)</i>	5'-AAC TTC AAC AAG CTC GTG ATG CC-3' 5'-GCT TCT AAA CAT CTC CTT ACA CGG G-3'	1.97
<i>Etv3 (l)</i>	5'-AAC TTC AAC AAG CTC GTG ATG CC-3' 5'-GAG AAT GGC TGT CTA GAG GTG GG-3'	1.96
<i>Trim30</i>	5'-AGC GAT CTC AGG AGC ACC G-3' 5'-TTC CAC AGA GCT CCC TGC AG-3'	1.94
<i>Csf3r</i>	5'-AGG GCT ATC TCA TTG AGT GGG-3' 5'-CTG TAA TTC TGT AGA GCT GAA AGG G-3'	1.81
<i>Bbx</i>	5'-CTT TTC TCA TCC GAT GGA TTC ACC-3' 5'-GAT GCC ACT GAA GAC ACT TCC G-3'	1.88
<i>Atp2a1</i>	5'-CCT CCT CCA TGT CTT TGA ACC G-3' 5'-TGA AGA TGC ATG GCT ATT GGG-3'	1.94
<i>Elavl4</i>	5'-ATC ATC ACC TCA CGC ATC CTG-3' 5'-TTG GCA AAC TTC ACA GTA ATC GG-3'	1.91
<i>Il17r</i>	5'-ACC ACA GCT GCT TTG ATG TCG-3' 5'-TGG TAT TTG AGA TTA CTG GGC AGG-3'	1.91
<i>Klk6</i>	5'-AAT GAC CTG ATG CTG CTC CG-3' 5'-TTT GGC ACA GTC CTC ATT AGG C-3'	1.85
<i>9830147J24Rik</i>	5'-AGA ATG ACT CGT GGA TCT TCG C-3' 5'-GAC CGT GAA GTG GAC TTT GCC-3'	2.00
<i>S100a9</i>	5'-CTC ATG GAC TGA TTA TGG ACA GGA C-3' 5'-ATG CAG CTT CTC ATG ACA GGC-3'	2.00
<i>F13a1</i>	5'-GGA TTC ATT TGG ATG GTC CTG G-3' 5'-GGA GTC ACT GGT CAT GCT GGC-3'	1.91
<i>Actb</i>	5'-TGA CGG GGT TCA CCC ACA CTG TGC CCA TCT A-3' 5'-CTA GAA GCA TTT GTG GTG GAC GAT GGA GGG-3'	1.83
<i>Hprt</i>	5'-ATG GTG GAA GCA CAG TTG GC-3' 5'-GCA GGT CAG CAA AGA ACT TAT AGC C-3'	1.91

previously described (15). Duplicate transfections were harvested after 24 h, and cell lysates were assayed for firefly luciferase activity using the Luciferase Reporter Assay System (Promega). The firefly luciferase activity of individual transfections was normalized against protein concentration measured using a bicinchoninic acid assay (Sigma-Aldrich). For some experiments, RAW264.7 cells were transfected in 10-cm tissue culture dishes as described above using linearized reporter constructs (10 μ g) as well as a plasmid carrying the neomycin resistance gene (5 μ g). Cells were selected for stable integration of plasmid DNA by culturing cells in RPMI 1640 medium supplemented with 350 μ g/ μ l G418 for 2–3 wk. Stably transfected cells were pooled and expanded, and 750,000 cells/ml were seeded into six-well plates in duplicate the day before stimulation. Cells were harvested 5 h after stimulation, and cell lysates were assayed as described above.

Nuclear extracts and EMSA

Nuclear extracts of RAW264.7 cells and BMM were prepared as described previously (4). Double-stranded oligonucleotides corresponding to the STAT6 or NF κ B elements were labeled with [α - 32 P]dGTP using Klenow DNA polymerase. Sequences of individual motifs are indicated in Figs. 5 and 6. The binding reaction contained 2.5 μ g of nuclear extract protein, 0.5 μ g of poly d(I/C), 20 mM HEPES (pH 7.9), 20 mM KCl, 1 mM DTT, 1 mM EDTA (pH 8.0), 5% glycerol, and 20 nmol of probe DNA in a final volume of 10 μ l. Antisera used in supershift analyses were added after 15 min, and samples were loaded onto polyacrylamide gels after incubation at room temperature for a total of 30 min. The buffers and running conditions used have been described previously. Gels were fixed in 5% acetic acid, dried, and autoradiographed.

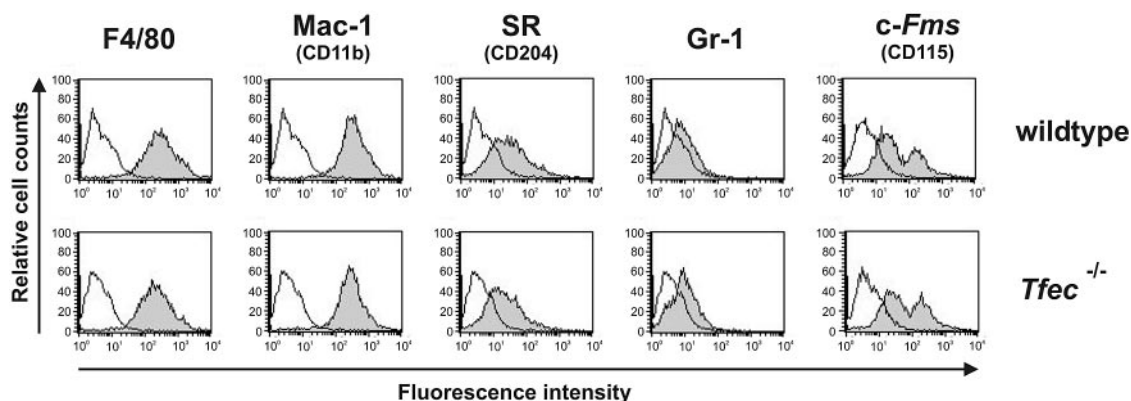


FIGURE 1. Flow cytometry. BMM from wild-type and *Tfec*-deficient mice (*Tfec*^{-/-}) were analyzed by flow cytometry for the expression of the indicated cell surface markers.

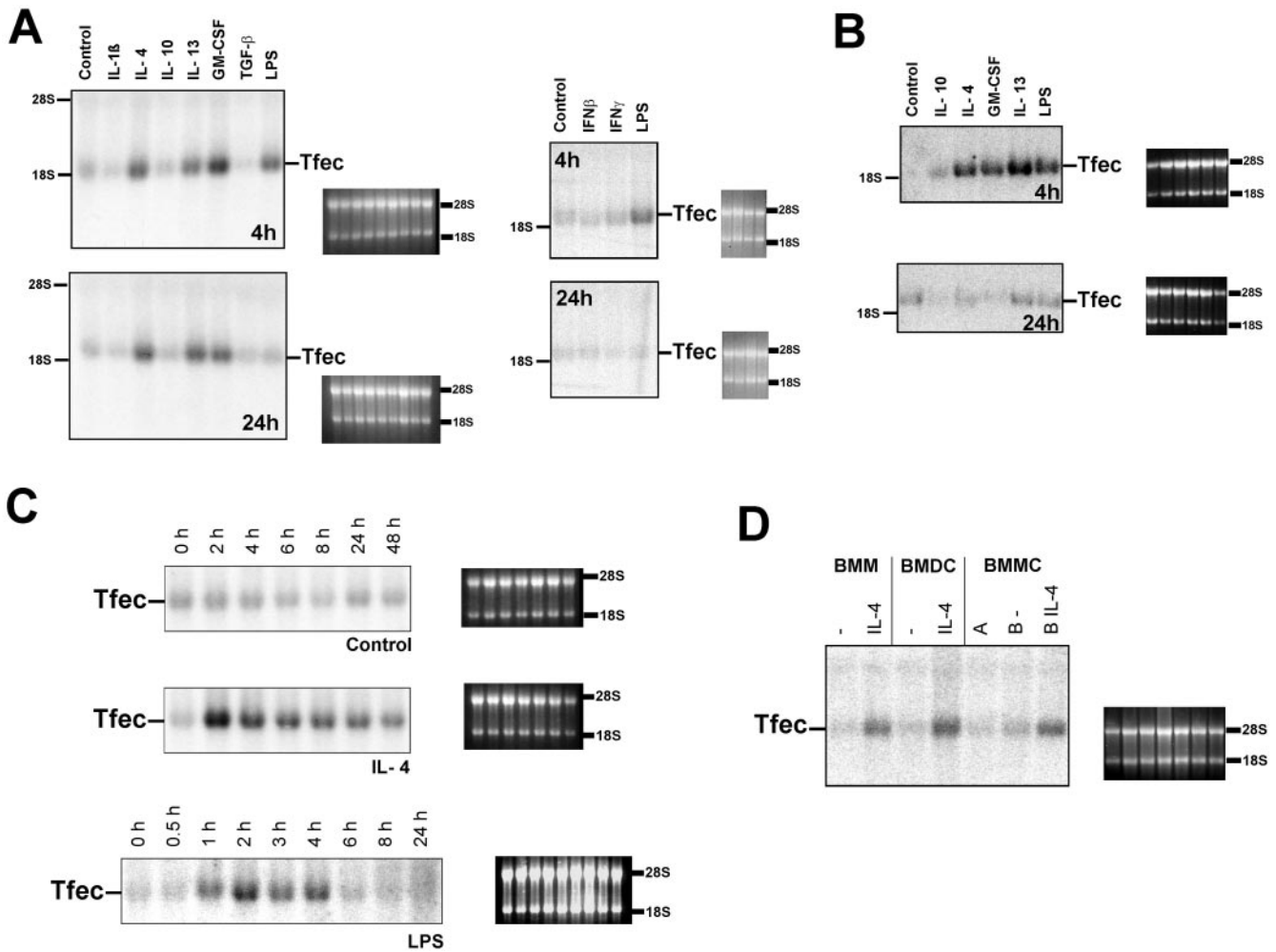


FIGURE 2. Northern analysis of inducible Tfec expression in macrophages. Total RNA was isolated from BALB/c BMM (A) and RAW264.7 cells (B) treated for 4 or 24 h with the indicated stimuli and untreated (C) or treated with IL-4 (10 ng/ml) or LPS (1 μ g/ml) at 0 h for the indicated time periods. D, Total RNA was isolated from BALB/c BMM, BMDC, and BMMC (cultured in the presence of CSF-1, GM-CSF, SCF/IL-4 (A) and SCF/IL-3 (B), respectively), untreated or treated with IL-4 for 4 h. The blots were hybridized with a 32 P-labeled Tfec cDNA. The ethidium bromide staining is shown as a control for mRNA loading.

Western blot analysis

The anti-Tfec rabbit polyclonal antiserum was raised against a GST fusion protein with the C-terminal aa 199–317 of Tfec as described previously (8). The antiserum showed no cross-reactivity against in vitro-translated rMiT proteins Mitf, Tfe3, and Tfeb. For the detection of Tfec, whole cells were lysed with SDS (1%) containing 1 mM Na_3VO_4 and a mixture of protease inhibitors (2 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 mM PMSF; Roche). To detect the G-CSFR, 10^6 cells were lysed with Nonidet P-40 (1%) lysis buffer containing the above mixture of phosphatase and proteinase inhibitors. Cell lysates were separated by SDS-PAGE and blotted on a polyvinylidene difluoride membrane. Blots were blocked overnight at 4°C with 5% milk powder/0.1% Tween 20/TBS and incubated with anti-Tfec (1/200), anti-G-CSFR (1/500), or anti- β -actin antisera (1/2000) and subsequently with HRP-conjugated goat anti-mouse Ab (DakoCytomation) diluted 1/2500. Bands were visualized using ECL reagents (Amersham Biosciences), scanned, and analyzed using a Molecular Dynamics personal densitometer (Amersham Biosciences).

Results

Normal phenotype of Tfec-deficient macrophages

Mice lacking Tfec develop normally and show no obvious histological alterations. Bone marrow cultures of both wild-type and Tfec-deficient animals gave rise to similar numbers of macrophages in the presence of CSF-1, and the resulting cells were morphologically indistinguishable (data not shown). A comparison of

wild-type and Tfec-deficient macrophages using flow cytometry revealed that marker molecules F4/80-Ag, scavenger receptor, CSF-1R (*c-Fms*), Mac-1, and Gr-1 were expressed at similar levels (Fig. 1). This may indicate a redundant role for Tfec in normal macrophage development.

Inducible expression pattern of Tfec in macrophages

Macrophages respond to a large number of environmental cues with sometimes marked phenotypical and functional changes. To investigate whether Tfec might be regulated during macrophage activation, we treated BMM (BALB/c) with a number of cytokines and proinflammatory stimuli to detect changes in Tfec mRNA expression. As shown in Fig. 2A, Tfec mRNA was markedly up-regulated by the Th2 cytokines IL-4 and IL-13, GM-CSF, and LPS. Several other mediators, including IL-1 β , TGF- β , IFN- β , and IFN- γ , had no marked effect on Tfec mRNA expression. Similar results were obtained using the macrophage cell line RAW264.7 (Fig. 2B) and in macrophages from a genetically different background (129Sv/J), with the exception of GM-CSF, which failed to induce Tfec expression in the macrophages from 129Sv/J mice (data not shown). The regulation by LPS and IL-4, the archetypal inducers of classically and alternatively activated macrophages, respectively, was further analyzed. As shown in

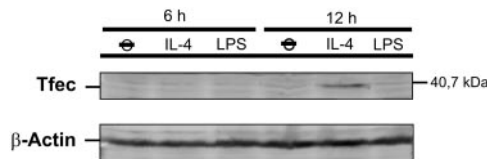


FIGURE 3. Expression of Tfec protein is up-regulated in IL-4-treated macrophages. Total cell lysates were prepared from BMM untreated or treated with IL-4 (10 ng/ml) or LPS (1 μ g/ml) at 0 h for the indicated time periods. Proteins were separated by SDS-PAGE and blotted. Western analysis was performed using a Tfec antiserum or an anti- β -actin mAb to control for equal loading.

Fig. 2B, LPS induced a rapid, but transient, up-regulation of Tfec mRNA expression, whereas IL-4 treatment resulted in a rapid, but long-lasting, increase in Tfec transcripts. Tfec mRNA induction was dose dependent in both cases (data not shown). In a separate study, one of the authors (D. A. Hume) and his colleagues in the FANTOM3 consortium performed CAGE start site analysis on BMM responding to LPS and CpG DNA (data not shown). The data confirm the rapid, transient induction of Tfec and showed that Tfeb is similarly expressed and regulated, whereas Tfe3 and Mitf are expressed constitutively and are not further inducible.

To test whether IL-4 induction was cell type-specific, we generated BMDC as well as mast cells and stimulated the cultures with IL-4. As shown in Fig. 2C, both myeloid cell types expressed Tfec mRNA levels comparable to macrophages, and Tfec was induced by IL-4 in both cases. No induction by IL-4, however, was observed in NIH-3T3 fibroblasts (data not shown).

To follow the expression of Tfec protein during macrophage activation, we generated a Tfec-specific antiserum that was subsequently used to detect Tfec in Western analyses. The signals

were generally below the background staining in untreated and 6-h stimuli-treated macrophages. A clear band with the expected m.w. was reproducibly detected in macrophages that were treated with IL-4 for 12 h (Fig. 3), but not in LPS-treated cells. Without a defined standard, we cannot imply that there is no basal or LPS-inducible Tfec protein in macrophages, but clearly, the levels are significantly elevated by IL-4.

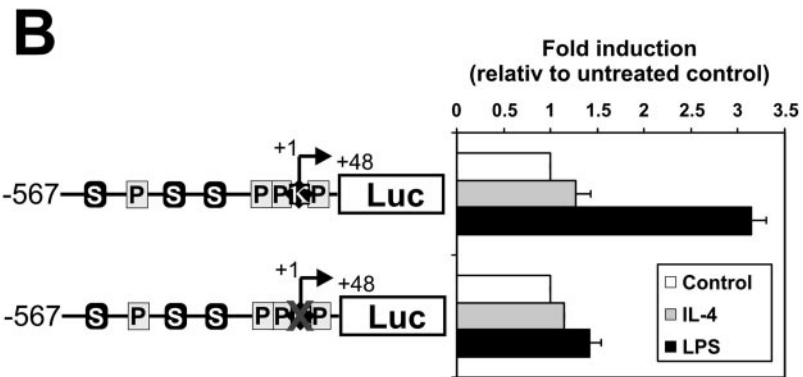
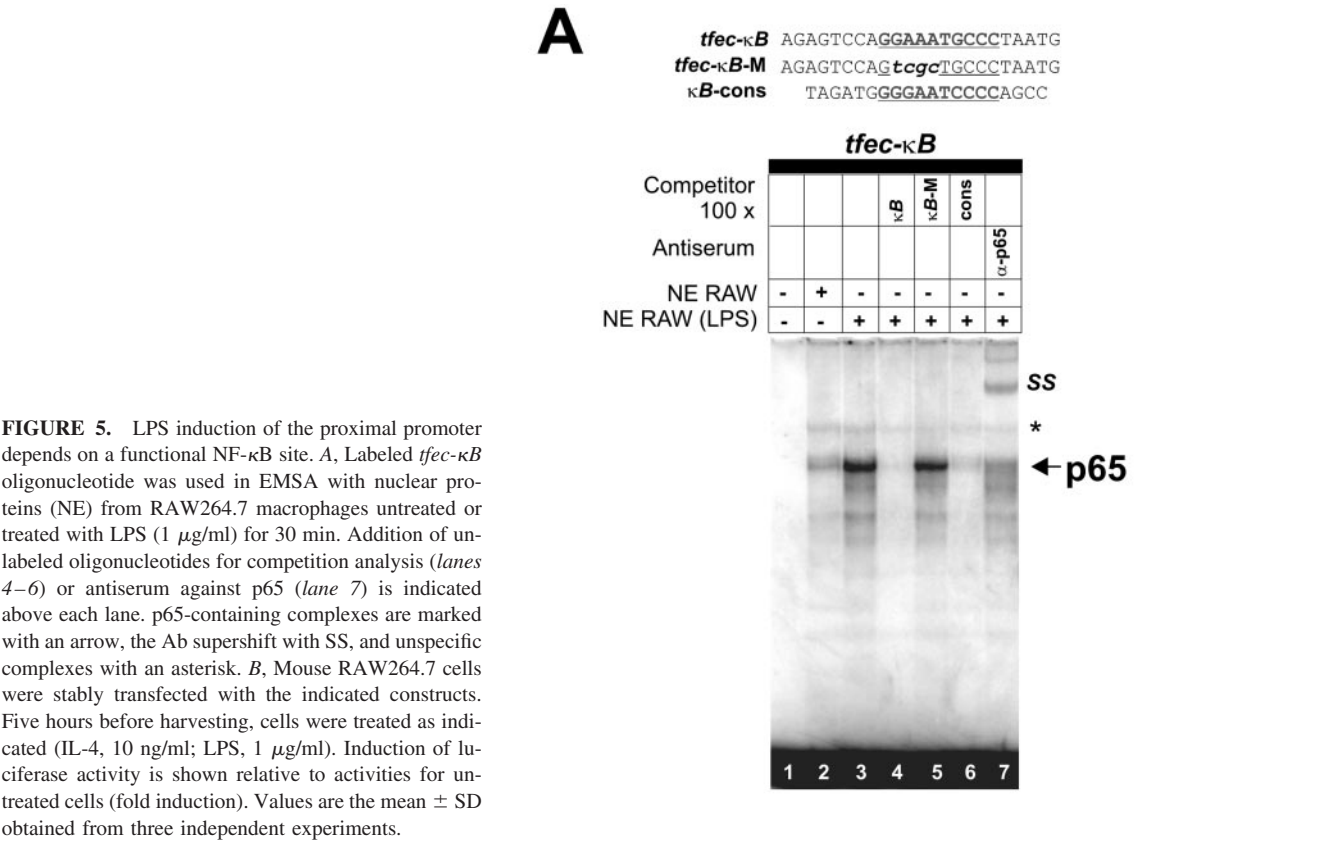
Analysis of LPS- and IL-4-mediated induction of Tfec

To analyze the regulatory mechanisms of LPS- and IL-4-induced up-regulation of Tfec, we performed an analysis of the proximal promoter of the Tfec gene. Initial transfection experiments indicated that a luciferase construct of the proximal promoter was responsive to LPS (but not IL-4) treatment in RAW264.7 cells. Sequence analysis of the proximal human, rat, and mouse Tfec promoter regions revealed the presence of a conserved NF- κ B motif in all three species (see Fig. 4). To determine the role of NF- κ B in Tfec induction by LPS, we performed gel-shift experiments using nuclear extracts from untreated and LPS-treated RAW264.7 macrophages. As shown in Fig. 5A, the NF- κ B motif formed specific and inducible complexes with the NF- κ B family member p65. To further confirm its role in LPS-mediated induction of promoter activity, we mutated the conserved NF- κ B motif in the previously described 615-bp promoter construct. As shown in Fig. 5B, mutation of the NF- κ B element completely abolished LPS-induced reporter activity.

In RAW264.7 macrophages, the proximal promoter of Tfec did not respond to IL-4 stimulation in stable (shown in Fig. 5B) and transient (data not shown) transfections despite the presence of three Stat6 consensus binding sites. However, as shown in Fig. 6B, specific and inducible binding of nuclear Stat6 to two sites within the proximal promoter was demonstrated in gel-shift experiments

FIGURE 4. Sequence alignment of human, rat, and mouse Tfec promoter sequences. The 5'-proximal mouse Tfec promoter was aligned with the corresponding rat and human promoter sequences. In humans, two additional promoter regions may exist. The region corresponding to both murine promoters precedes exon 1b of the human gene (7). Putative binding sites for several transcription factors that were identified using the MatInspector database (Genomatix) are indicated.





using nuclear extracts from BMM. Welch et al. (16) reported that RAW264.7 macrophages are defective in their IL-4 signaling pathway in transfection assays. As shown in Fig. 6A, the IL-4 responsiveness was restored by cotransfecting an expression plasmid for Stat6 as previously described by Welch et al. (16). The *Tfec*-promoter construct was induced to a similar extent as a multimerized Stat6 site from the human 12/15-lipoxygenase promoter (4×STAT6-TK). Whereas the mutation of the outer (o) Stat6 element had no impact on the reporter activity of the *Tfec* promoter construct, mutation of the middle (m) and the inner (i) site caused a marked or complete reduction of IL-4-induced activation, respectively (Fig. 6A). To unequivocally demonstrate that Stat6 was required for IL-4 induction of *Tfec* transcription, we stimulated macrophages from Stat6-deficient mice with IL-4 and compared *Tfec* expression with corresponding wild-type macrophages. As shown in Fig. 6C, Stat6-deficient macrophages did not induce *Tfec* mRNA expression after treatment with IL-4.

Global analysis of IL-4-induced gene expression in BMM

The above data indicated that *Tfec* might act as a secondary transcription factor of the IL-4 signaling cascade in macrophages downstream of Stat6. To characterize the IL-4-induced expression profile of alternatively activated macrophages and to identify those genes that are regulated in a *Tfec*-dependent fashion, we compared the transcriptome of IL-4-treated wild-type and *Tfec*-deficient mouse macrophages using cDNA and oligonucleotide microarrays. Because the protein product of the *Tfec* gene was induced with delayed kinetics, we analyzed the transcriptome of macrophages (both wild type and *Tfec* deficient) after 16-h treatment with IL-4. The list of genes that were regulated by IL-4 in wild-type macrophages contained a number of genes that were previously described as IL-4 regulated, including, e.g., arginase 1 (*Arg1*), decan-1 (*Clec3f12*), and Ym1 (*Chi3l3*) as well as *Tfec*. Microarray results of wild-type macrophages are summarized in Fig. 7, A and B. In comparison, most genes were almost identically regulated by

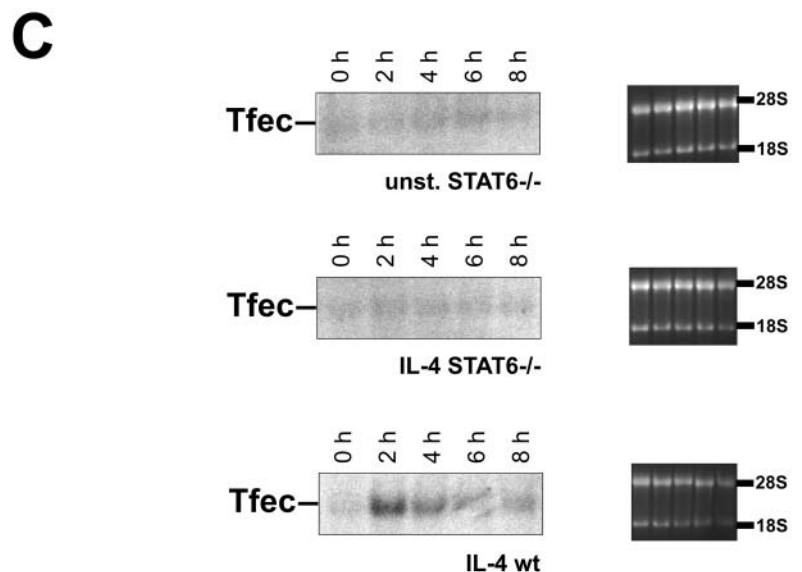
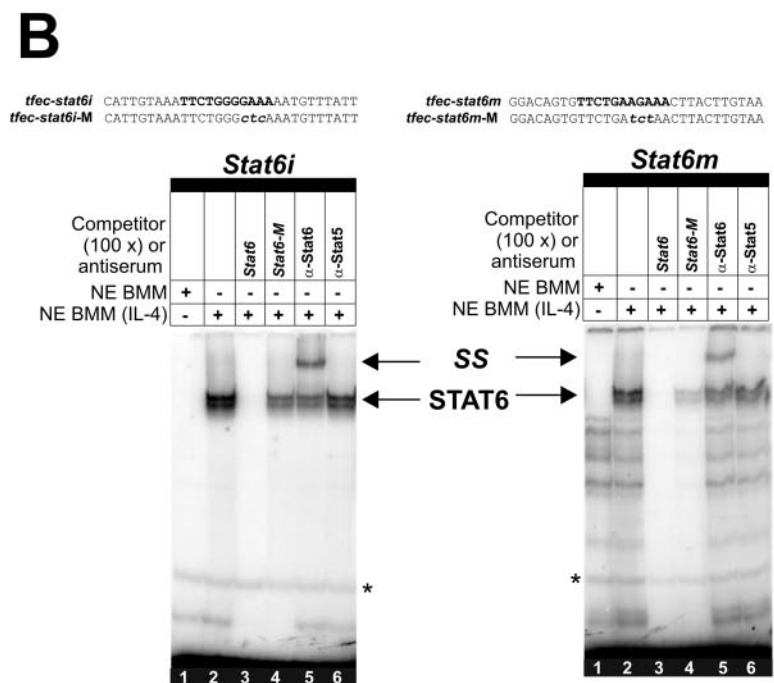
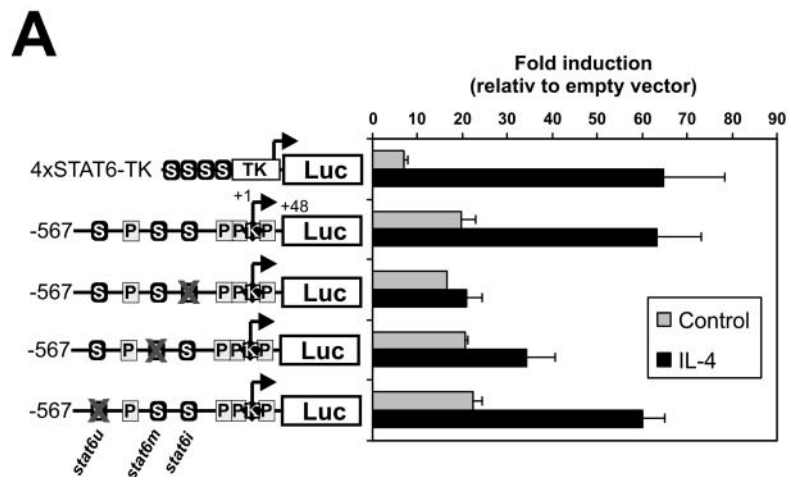


FIGURE 6. IL-4-induction of Tfec mRNA is STAT6 dependent. *A*, Mouse RAW264.7 cells were transiently transfected with the indicated constructs as well as a Stat6 expression plasmid. Sixteen hours before harvesting, cells were treated with IL-4 (20 ng/ml). Induction of luciferase activity is shown relative to the activity of the empty pGL3-B vector. Values are the mean \pm SD obtained from three independent experiments. *B*, Labeled *tfec-Stat6i* and *tfec-Stat6m* oligonucleotides were used in EMSA with nuclear proteins (NE) from BMM untreated or treated with IL-4 (10 ng/ml) for 2 h. Addition of unlabeled oligonucleotides for competition analysis (*lanes 3 and 4*) or antisera against Stat factors (*lanes 5 and 6*) is indicated above each lane. Stat6-containing complexes are marked with an arrow, Ab supershifts with SS, and unspecific complexes with an asterisk. *C*, Northern analysis of IL-4-inducible Tfec expression in Stat6-deficient macrophages. Total RNA was isolated from wild-type (wt) and Stat6-deficient (Stat6^{-/-}) BMM untreated or treated with IL-4 (10 ng/ml) at 0 h for the indicated time periods. The blots were hybridized with a ³²P-labeled Tfec cDNA. The ethidium bromide staining is shown as a control for mRNA loading.

A Up-regulated by IL-4

Gene		fold-induction
eosinophil-associated, ribonuclease A family, member 11	Ear11	1552.1
arginase 1, liver	Arg1	294.1
resistin like alpha	Retnla	274.4
macrophage galactose N-acetyl-galactosamine specific lectin 2	Mgl2	73.5
PDZ and LIM domain 1 (elfin)	Pdlm1	55.7
FMS-like tyrosine kinase 1 (VEGF receptor)	Flt1	48.9
cadherin 1 (E-cadherin)	Cdh1	42.2
aminoacylase 1	Acy1	24.3
diphtheria toxin receptor	Dtr	24.2
suppressor of cytokine signaling 1	Socs1	22.6
purinergic receptor P2Y, G-protein coupled 1	P2ry1	21.1
early growth response 2	Egr2	19.0
interleukin 4 induced 1	Il4i1	18.4
coagulation factor X	F10	16.8
homeo box, msh-like 3	Msx3	16.0
macrophage galactose N-acetyl-galactosamine specific lectin 1	Mgl1	13.9
cationic amino acid transporter 2	Slc7a2	13.3
claudin 11	Cldn11	13.0
dectin-1	Clec3f12	12.1
chemokine (C-C motif) ligand 7 (Mcp3)	Ccl7	11.3
fibronectin 1	Fn1	9.8
phospholipase A2, group X1IA	Pla2g12a	9.8
leukotriene B4 receptor 1	Ltb4r1	9.2
cystatin F (leukocystatin)	Cst7	9.2
aquaporin 3	Aqp3	8.6
plexin domain containing 2	Plxdc2	8.0
proviral integration site 3	Pim3	7.1
chitinase 3-like 3 (Ym1)	Chi3l3	6.5
coagulation factor VII	F7	6.5
caspase 6	Casp6	6.1
basic helix-loop-helix domain containing, class B2	Bhlhb2	6.1
cytokine inducible SH2-containing protein	Cish	5.7
adaptor protein with pleckstrin homology and src	Aps	5.5
megakaryocyte-associated tyrosine kinase	Matk	5.4
tubulin, alpha 8	Tuba8	5.3
ATP-binding cassette, sub-family D (ALD), member 2	Abcd2	4.9
programmed cell death 1 ligand 2 (B7DC)	Pdcd1lg2	4.9
phafin1	Plekhl1	4.9
sialyltransferase 8 (alpha-2, 8-sialyltransferase) A	Siat8a	4.8
histocompatibility 2, class II, locus DMA	H2-DMA	4.6
CD83 antigen	Cd83	4.6
RAS guanyl releasing protein 1	Rasgrp1	4.4
macrophage scavenger receptor 2	Ms2	4.3
nuclear factor, interleukin 3, regulated	Nfil3	4.3
interleukin 1 receptor-like 1	Il1rl1	4.2
class II transactivator	C2ta	4.0
epoxide hydrolase 1, microsomal	Ephx1	4.0
transferrin receptor	Tfrc	4.0
kruppel-like factor 4 (gut)	Klf4	4.0
glutaminyl-peptide cyclotransferase (glutaminy cyclase)	Qpct	4.0
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	Atp6v0a1	3.8
WD repeat domain 9	Wdr9	3.8
receptor (calcitonin) activity modifying protein 1	Ramp1	3.7
transient receptor potential cation channel, subfamily V, member 4	Trpv4	3.7
programmed cell death 1 ligand 1 (B7H1)	Pdcd1lg1	3.7
glypican 1	Gpc1	3.7
colony stimulating factor 2 receptor, beta 1, low-affinity	Csf2rb1	3.7
protein tyrosine phosphatase, receptor type, D	Ptpd	3.5
chemokine (C-C motif) ligand 12	Ccl12	3.5
kallikrein 11	Klk11	3.5
transcription factor EC	Tcfec	3.5
alanyl (membrane) aminopeptidase	Anpep	3.5
angiotensin-like 2	Angptl2	3.5
O-acyltransferase (membrane bound) domain containing 2	Oact2	3.5
hairynhancer-of-split related with YRPW motif 1	Hey1	3.5
Fc receptor, IgG, low affinity IIb	Fcgr2b	3.3
interleukin 11 receptor, alpha chain 2	Il11ra2	3.2
integrin alpha X	Itgax	3.2
gap junction membrane channel protein alpha 1	Gja1	3.2
CD164 antigen	Cd164	3.2
asialoglycoprotein receptor 2	Asgr2	3.2
TAR (HIV) RNA binding protein 2	Tarbp2	3.2
mannose receptor, C type 1	Mrc1	3.2
CD200 receptor 1	Cd200r1	3.2
IBR domain containing 3	Ibrdc3	3.2
colony stimulating factor 3 receptor(granulocyte)	csf3r	3.2
basic transcription element binding protein 1	Bteb1	3.0
DDHD domain containing 1	Ddhd1	3.0
endothelin 1	Edn1	3.0
Von Willebrand factor homolog	Vwf	3.0
hemochromatosis	Hfe	3.0
tyrosine kinase, non-receptor, 2	Tnk2	3.0
activating transcription factor 5	Atf5	3.0
chloride channel 5	Cln5	3.0
calcium channel, voltage-dependent, alpha 1F subunit	Cacna1f	3.0
la-associated invariant chain	Ii	3.0
histocompatibility 2, class II, locus Mb1	H2-DMb1	3.0
suppressor of cytokine signaling 2	Socs2	3.0
ets variant gene 3	Etv3	3.0
CD24a antigen	Cd24a	3.0
prostaglandin-endoperoxide synthase 1	Ptas1	3.0

C Higher in Tfec-/- after 16 h IL-4

Gene	fold-induction over wt.
RIKEN cDNA 9830147J24 gene	9830147J24Rik 14.0
zinc finger protein 119	Zfp119 11.3
interleukin 17 receptor	Il17r 6.9
ELAV-like 4 (Hu antigen D)	Elavl4 5.5
A730063E03	--- 5.4
B-cell CLL/lymphoma 11A	Bcl11a 4.8
jumonji, AT rich interactive domain 1A (Rbp2 like)	Jarid1a 3.5
interleukin 6	Il6 3.4
lectin, galactose-binding, soluble 2	Lgals2 3.3
S100 calcium binding protein A9 (calgranulin B)	S100a9 2.4
coagulation factor XIII, A1 subunit	F13a1 2.4

B Down-regulated by IL-4

Gene		fold-repression
interferon-induced protein 44	Ifi44	9.8
DEAD-box polypeptide 6 (Rck)	Ddx6	9.5
paired immunogloblin-like type 2 receptor beta	Pilrb1	8.0
G protein-coupled receptor 84	Gpr84	8.0
2'-5' oligoadenylate synthetase-like 2	Oasl2	7.5
lymphocyte antigen 6 complex, locus A	Ly6a	7.0
toll-like receptor 2	Tlr2	6.5
histocompatibility 2, T region locus 24	H2-T24	6.1
chemokine (C-X-C motif) ligand 10 (Ipl10)	Cxcl10	6.1
T-cell specific GTPase	Tgtp	5.7
guanylate nucleotide binding protein 4	Gbp4	5.7
chemokine (C-X-C motif) ligand 1 (Gro1)	Cxcl1	4.9
thymidylate kinase family LPS-inducible member	Tyki	4.9
sialyltransferase 10 (alpha-2,3-sialyltransferase VI)	Siat10	4.9
interleukin 13 receptor, alpha 1	Il13ra1	4.9
integrin alpha 6	Itga6	4.9
chemokine (C-X-C motif) ligand 16	Cxcl16	4.9
interferon-induced protein with tetratricopeptide repeats 2	Ifit2	4.9
mincle	Clec3f9	4.8
high mobility group AT-hook 2	Hmga2	4.7
guanylate nucleotide binding protein 2	Gbp2	4.7
guanylate nucleotide binding protein 1	Gbp1	4.6
myosin X	Myo10	4.6
calcitonin receptor-like	Calcr1	4.6
tumor necrosis factor	Tnf	4.6
semaphorin H	Sema4d	4.6
CD14 antigen	Cd14	4.6
myeloid cell nuclear differentiation antigen	Mnda	4.3
cytotoxic T lymphocyte-associated protein 2 beta	Ctia2b	4.5
ganglioside-induced differentiation-associated-protein 10	Gdap10	4.0
serine (or cysteine) proteinase inhibitor, clade B, member 6b	Serpinc6b	4.0
interferon induced transmembrane protein 6	Ifitm6	4.0
apolipoprotein B editing complex 1	Apoecb1	4.0
paired immunogloblin-like type 2 receptor alpha	Pilra	4.0
chemokine (C-X-C motif) ligand 2 (Mip2)	Cxcl2	4.0
immune-responsive gene 1	Irg1	4.0
receptor (calcitonin) activity modifying protein 2	Ramp2	3.8
solute carrier family 20, member 1	Sic20a1	3.8
phosphatase and tensin homolog	Pten	3.7
FBJ osteosarcoma oncogene	Fos	3.7
2'-5' oligoadenylate synthetase-like 1	Oasl1	3.7
macrophage C-type lectin	Clec3f8	3.7
carboxypeptidase D	Cpd	3.7
interferon, alpha-inducible protein	G1p2	3.7
dedicator of cytokinesis 7	Dock7	3.6
caveolin 2	Cav2	3.5
hemopoietic cell kinase	Hck	3.5
nuclear receptor interacting protein 1	Nrip1	3.4
tripartite motif protein 30	Trim30	3.4
regulator of G-protein signaling 2	Rgs2	3.3
Mdi-1	Clec3f5	3.2
ankyrin repeat domain 28	Ankrd28	3.2
2'-5' oligoadenylate synthetase 2	Oas2	3.2
Rho GTPase activating protein 24	Arhgap24	3.2
delta/notch-like EGF-related receptor	Dner	3.1
prostaglandin E receptor 4 (subtype EP4)	Ptger4	3.1
chemokine (C-X-C motif) ligand 14 (Brak)	Cxcl14	3.1
sialyltransferase 9	Siat9	3.0
interferon activated gene 203	Ifi203	3.0
dsRNA-activated kinase	Prkr	3.0
MARCKS-like protein	Mlp	3.0
protein phosphatase 2, regulatory subunit A (PR 65), beta	Ppp2r1b	3.0
signal transducer and activator of transcription 1	Stat1	3.0
adrenergic receptor, beta 2	Adrb2	3.0
acyl-CoA synthetase long-chain family member 1	Acsl1	3.0
PFTAIRE protein kinase 1	Ptk1	3.0
cyclin D2	Ccnd2	3.0
chromosome condensation 1-like	Chc11	3.0
sphingomyelin phosphodiesterase, acid-like 3B	Smpd3b	3.0
Fc receptor, IgG, high affinity I	Fcgr1	3.0
dectin-2	Clec3f10	3.0
immunoglobulin heavy chain 6 (heavy chain of IgM)	Igh-6	3.0

D Higher in Tfec-/-

Gene		fold-induction over wt
kallikrein 6	Kik6	13.3
A730063E03	---	3.9
calcium binding protein, intestinal	Cai	2.3

E Lower in Tfec-/-

Gene	fold-reduction over wt	
interleukin 6	il6	14.3
B-cell CLL/lymphoma 11A (zinc finger protein)	Bcl11a	5.4
solute carrier family 25 (mitochondrial carrier, brain), member 14	Sic25a14	2.5
bobby sox homolog (Drosophila)	Bbx	2.2
lectin, galactose-binding, soluble 2	Lgals2	2.1

F Lower in Tfec-/- after 16 h IL-4

Gene		fold-reduction over wt
kallikrein 6	Kik6	14.0
bobby sox homolog (Drosophila)	Bbx	11.3
procollagen, type VI, alpha 3	Col6a3	6.9
B-cell CLL/lymphoma 11A (zinc finger protein)	Bcl11a	5.5
ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	Atp2a1	5.4
insulin-like growth factor I receptor	Igf1r	4.8
proteasome (prosome, macropain) subunit, alpha type 6	Psm6	3.5
DNA segment, Chr 5, Brigham & Women's Genetics 0834	D5Bwg0834e	2.4
colony stimulating factor 3 receptor (granulocyte)	csf3r	2.1

IL-4 in Tfec-deficient macrophages. Microarray signals of only a small number of genes (listed in Fig. 7, C–F) were significantly altered in mutant macrophages.

To further validate the results obtained by microarray analysis, we analyzed the expression of several genes that were either regulated in wild-type macrophages or affected in Tfec-deficient animals using LightCycler real-time PCR. In wild-type macrophages we focused on transcription factors that were novel targets of IL-4, including early growth response 2 (*Egr2*, Krox20), Krueppel-like factor 4 (*Klfl4*, Gklf), *ets* variant gene 3 (*Etv3*, PE1/Mets), and Trim30. As shown in Fig. 8A–E, all four factors showed an expression pattern similar to the one observed using microarrays.

The effect of Tfec deficiency on genes identified by microarray analysis was generally less reproducible by real-time PCR analysis. Expression profiles of several genes (*S100a9*, see Fig. 8G; *IL17r*, see Fig. 8I; *Atp2a1*, *Elavl4*, *F13a1*, and *9830147j24Rik*, data not shown) as determined by LightCycler PCR in three biologically independent samples were not significantly altered in Tfec-deficient macrophages. Only a small number of genes were significantly and reproducibly altered in Tfec-deficient macrophages, including the previously uncharacterized transcription factor bobby sox homologue (*Bbx*, shown in Fig. 8F), the G-CSFR (*Csf3r*, shown in Fig. 8J), and the serine protease kallikrein 6 (*Klk6*, shown in Fig. 8H). Because a regulated expression of the G-CSFR in macrophages has not previously been reported, this gene was chosen for detailed study. Based upon the array data (Fig. 7F), the level of G-CSFR mRNA in IL-4-treated, Tfec-deficient macrophages was significantly reduced (2-fold) compared with that in IL-4-treated, wild-type macrophages. To confirm the Tfec-dependent regulation of the *Csf3r* gene, real-time PCR was performed using wild-type and Tfec-deficient BMM untreated or treated with IL-4 for up to 16 h. As shown in Fig. 8J, the IL-4-dependent induction of the *Csf3r* gene was markedly reduced in Tfec-deficient macrophages. In contrast, the induction of another known IL-4 target gene in macrophages, arginase 1 (*Arg1*), was unaffected by the Tfec-mutation (Fig. 8K). To investigate whether the G-CSFR protein was also induced by IL-4 in a Tfec-dependent fashion, we performed Western analyses using membrane lysates from wild-type and Tfec-deficient BMM untreated or treated with IL-4 for up to 30 h. As shown in Fig. 8, L and M, G-CSFR protein was indeed induced after 20-h treatment with IL-4, and the extent of induction was markedly reduced in Tfec-deficient macrophages.

Discussion

The identity and function of differentiated cells are determined by the concerted action of ubiquitous and, most importantly, cell type-specific transcription factors. We showed previously that the bHLH transcription factor Tfec is specifically expressed in mouse mononuclear phagocytes, indicating a possible role for Tfec in the development or function of these cells (4). The present report further investigates the regulation and function of Tfec in activated macrophages. Upon stimulation, the most striking effects on Tfec levels were observed in IL-4-treated, alternatively activated macrophages. Both mRNA and protein were markedly up-regulated, suggesting a role for Tfec in regulating the alternative activation program in macrophages or dendritic cells. We also identified the first three putative target genes of Tfec in IL-4-treated macrophages, which include the *Csf3r* gene encoding the G-CSFR.

Based on mRNA expression studies of several macrophage and nonmacrophage cell types and an initial characterization of the proximal mouse Tfec promoter, our previous studies indicated a restricted expression of the mouse transcription factor Tfec in macrophages (4). The TATA-less promoter of Tfec shares features with many known macrophage-specific promoters and preferentially directed luciferase expression in the RAW264.7 macrophage cell line in transient transfection assays. Tfec expression probably depends on the myeloid and B cell-specific transcription factor PU.1, because several PU.1 motifs identified in the Tfec promoter bound PU.1 under in vitro conditions, and coexpression of PU.1 induced luciferase activity of the Tfec promoter in transfected NIH-3T3 fibroblasts (4). Mice lacking Tfec are indistinguishable from their wild-type littermates; they are viable and fertile, normally pigmented, have normal eyes and mast cells, and show no osteopetrosis, indicating that other MiT family members may compensate for Tfec deficiency under normal standard conditions (10). The normal phenotype of Tfec-deficient mice is surprising, because the expression pattern of Tfec is highly cell type specific. In humans, according to two reference databases for gene expression (LSBM: www.lsbm.org/site_e/database/index.html) or GNF SymAtlas: (symatlas.gnf.org)), TFEC mRNA expression appears even more macrophage restricted than many other macrophage markers, including PU.1. Given that the effect of Mitf mutation in mice is dependent upon genetic background, it is possible that Tfec deficiency would have a greater effect if backcrossed to distinct inbred strains.

In this study we considered the possibility that Tfec functions not in macrophage differentiation, but in some function associated with innate or acquired immunity. We studied the modulation of Tfec expression in macrophages treated with different pro- and anti-inflammatory mediators and found that Tfec expression at both the mRNA and protein levels is strongly induced in mouse macrophages by the Th2 cytokine IL-4. IL-4 also induced Tfec expression in two other PU.1-expressing cell types: myeloid BMDC and BMMC. As an important role for the MiT family member microphthalmia transcription factor (Mitf) has been demonstrated in mast cell biology, it will be interesting to study the role of Tfec in mast cells treated with IL-4. The apparent delay in Tfec protein expression in IL-4-treated macrophages and the absence of detectable Tfec protein in LPS-stimulated macrophages despite the up-regulation of Tfec mRNA may indicate that IL-4 positively regulates Tfec at the level of protein stability in addition to the transcriptional level.

IL-4 (and IL-13) are generally produced in Th2-type responses, particularly in allergic, cellular, and humoral responses to extracellular and parasitic pathogens (17). They are known to induce an alternative activation program in macrophages that is distinct from the classical activation induced by the Th1 cytokine IFN- γ (13). Recently, it was demonstrated that IL-4R α signaling and hence the generation of alternatively activated macrophages are critical for host survival during schistosomiasis (18). The alternative activation program includes the up-regulation of several receptors involved in phagocytosis (e.g., mannose receptor (19) or dectin-1 (20)) and Ag presentation (e.g., MHC class II molecules) (21). Alternatively activated macrophages are known to produce a distinct set of chemokines (e.g., CCL17 and CCL22) (22, 23) and

FIGURE 7. Microarray analysis of IL-4-dependent and independent gene regulation in wild-type and Tfec-deficient macrophages. A and B, Microarray detection of transcripts that increased (A) or decreased (B) at least 3-fold by wild-type BMM in response to recombinant mouse IL-4 (10 ng/ml). C–F, Comparison of transcript levels from wild-type (wt) and Tfec-deficient (Tfec^{−/−}) BMM either untreated (D and E) or treated with IL-4 for 16 h (C and F). Transcripts that were further analyzed by real-time PCR are highlighted.

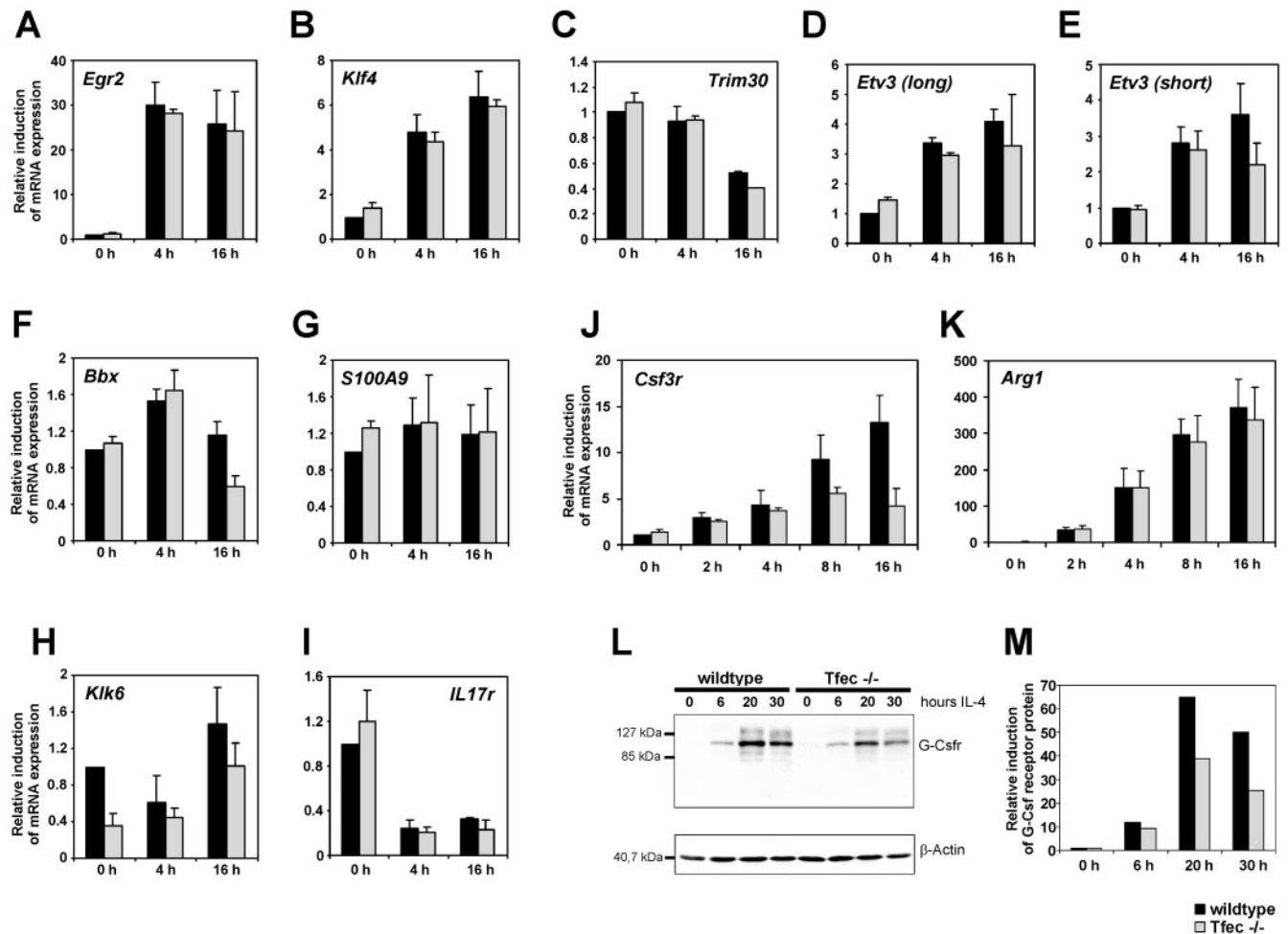


FIGURE 8. Comparative analysis of gene regulation on single gene level in wild-type and Tfec-deficient macrophages. A–K, Real-time quantitative RT-PCR (LightCycler) for the indicated genes was performed on cDNAs prepared from wild-type (wt) and Tfec-deficient (Tfec^{-/-}) BMM, either untreated or treated with recombinant mouse IL-4 (10 ng/ml) at 0 h for the indicated time periods. LightCycler results were normalized to housekeeping genes *Hprt* (A–I) or *Actb* (J and K) expression. Data represent the mean \pm SD of three independent preparations of BMM. L, Western analysis of G-CSFR protein levels in membrane extracts from untreated or IL-4-treated wild-type (wt) and Tfec-deficient (Tfec^{-/-}) BMM. β -Actin was stained to control for equal loading. M, Densitometric evaluation of G-CSFR protein expression. Signal intensities were normalized against β -actin, and values are relative to the band intensity of untreated wild-type macrophages.

intracellular enzymes (e.g., arginase 1) (24), and responses to microbial stimulation are different from classically activated macrophages in terms of cytokine release and production of reactive oxygen species (13). Our analysis of Tfec function entailed the first global analysis of IL-4-induced gene regulation in alternatively activated macrophages. We identified most of the previously characterized IL-4 target genes in macrophages and, in addition, characterized a new set of genes that may play a role in the biology of alternatively activated macrophages.

As expected, many of the identified genes were inversely regulated in IL-4-stimulated compared with IFN- γ -activated macrophages, including *Socs1* and *Socs2* (up-regulated by IL-4), *Stat1* (down-regulated by IL-4), and a large number of LPS- or IFN-inducible genes, e.g., *Ip10*, *TLR2* (*Tlr2*), dsRNA-activated kinase (PKR, *Prkr*), or *Cd14* (all down-regulated by IL-4).

A number of IL-4 target genes in macrophages have previously been identified on dendritic cells, including the B7 family members B7DC (*Pdcd1lg2*) and B7H1 (*Pdcd1lg1*), and the Cd83 Ag. Interestingly, IL-4 also induced three endothelial cell markers in macrophages: E-cadherin (*Cdh1*), vascular endothelial growth factor receptor (*Flt1*), and von Willebrand factor (*Vwf*). Expression of these markers was previously noted in human immature dendritic

cells that develop into endothelial-like cells when cultured in the presence of angiogenic growth factors (25).

Notably, we identified several transcription factors that are induced by IL-4, including, e.g., msh-like homeobox gene 3 (*Msx3*), early growth response 2 (*Egr2*, Krox20), bHLH transcription factor STRA13/Dec1 (*Bhlhb2*), IL-3-induced NF (*Nfil3*), Krueppel-like factor 4 (*Klf4*, Gklf), basic transcription element-binding protein 1 (*Bteb1*), and *ets* variant gene 3 (*Etv3*, PE1/Mets). It is conceivable that these transcription factors serve as secondary regulators of IL-4 signaling in macrophages and in some measure complement a Tfec deficiency. STRA13/Dec1, in particular, is another E box-binding protein that has been implicated in the regulation of STAT3 activity (26). It will be interesting to analyze their contributions to the phenotype of alternatively activated macrophages. In addition to Th2-type environments, tumors can induce an alternative activation macrophage phenotype (27). Tfec was originally cloned from a rat chondrosarcoma; however, no expression was detected in the respective chondrosarcoma cell line (5). It is therefore conceivable that Tfec is also induced in tumor-associated macrophages. The same might also be true for the above-mentioned transcription factors. Clearly, further studies are needed

to elucidate the role of these factors in tumor-associated macrophages.

To identify potential target genes of IL-4-induced Tfec in alternatively activated macrophages, we compared the expression profiles of IL-4-treated macrophages from wild-type and Tfec-deficient mice. Our LightCycler real-time PCR results indicate that only a small set of genes is differentially regulated, including the bobby sox homologue (*Bbx*), *Etv3* (short variant), G-CSFR (*Csf3r*), and kallikrein 6 (*Klk6*). Apparently, macrophages are largely able to compensate for Tfec deficiency. It needs to be clarified whether Tfec directly or indirectly regulated the above genes.

The expression profile of the G-CSFR gene was further analyzed. Up-regulation of the G-CSFR in alternatively activated macrophages has not been previously described and represents an interesting and potentially relevant finding in itself. It is clear that G-CSF is an essential factor for neutrophil development (28), but the actions of this cytokine on mononuclear phagocytes are not well defined. Low level expression of G-CSFR on mouse macrophages (29) as well as on human monocytes (30) has been demonstrated. In humans, the administration of G-CSF increased the expression of CR1, CR3, FcRI, and FcRIII on monocytes (31). Other studies indicate that G-CSF attenuates monocytic IL-12 and TNF release upon stimulation with LPS (30). Studies of G-CSF-deficient peritoneal mouse macrophages demonstrated a strong effect of G-CSF deficiency on macrophage activities and numbers (32). Our results show that the G-CSFR is strongly induced on macrophages by the Th2 cytokine IL-4, suggesting that G-CSF might specifically act on alternatively activated macrophages. It was also shown that G-CSF augments IL-4 secretion by T lymphocytes (33), indicating a role for G-CSF in Th2 immune responses. It is possible that the neutrophil growth factor G-CSF plays an additional, but as yet unrecognized, role in the biology and function of alternatively activated macrophages. A secondary issue that arises from a role for Tfec in G-CSFR production is that Tfec might have a role in granulocyte differentiation. Many granulocyte promoters also possess PU.1 sites, and PU.1 deficiency also ablates granulocyte production in mice (34, 35). Although the Tfec-deficient mice are not grossly granulocyte deficient, a detailed study of this lineage might reveal another role for Tfec.

In conclusion, we provide a global analysis of transcripts expressed by IL-4-treated mouse macrophages. We show that Tfec, along with several other transcription factors, is a Stat6-dependent target of IL-4. Tfec deficiency appears to have a mild impact on IL-4-induced gene expression, affecting only a few genes. Interestingly, Tfec participates in the IL-4 induction of the G-CSFR that is strongly induced on alternatively activated macrophages. Our results will inform further studies of the impact of the newly identified IL-4 targets as well as Tfec and G-CSFR up-regulation in macrophages in both tumor and Th2-type environments.

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Disclosures

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References

- Friedman, A. D. 2002. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* 21: 3377–3390.
- Nagamura-Inoue, T., T. Tamura, and K. Ozato. 2001. Transcription factors that regulate growth and differentiation of myeloid cells. *Int. Rev. Immunol.* 20: 83–105.
- Dahl, R., and M. C. Simon. 2003. The importance of PU.1 concentration in hematopoietic lineage commitment and maturation. *Blood Cells Mol. Dis.* 31: 229–233.
- Rehli, M., A. Lichanska, A. I. Cassady, M. C. Ostrowski, and D. A. Hume. 1999. TFEC is a macrophage-restricted member of the microphthalmia-TFE subfamily of basic helix-loop-helix leucine zipper transcription factors. *J. Immunol.* 162: 1559–1565.
- Zhao, G. Q., Q. Zhao, X. Zhou, M. G. Mattei, and B. de Crombrughe. 1993. TFEC, a basic helix-loop-helix protein, forms heterodimers with TFE3 and inhibits TFE3-dependent transcription activation. *Mol. Cell. Biol.* 13: 4505–4512.
- Chung, M. C., H. K. Kim, and S. Kawamoto. 2001. TFEC can function as a transcriptional activator of the nonmuscle myosin II heavy chain-A gene in transfected cells. *Biochemistry* 40: 8887–8897.
- Kuiper, R. P., M. Schepens, J. Thijssen, E. F. Schoenmakers, and A. G. van Kessel. 2004. Regulation of the Mitf/TFE bHLH-LZ transcription factors through restricted spatial expression and alternative splicing of functional domains. *Nucleic Acids Res.* 32: 2315–2322.
- Mansky, K. C., S. Sulzbacher, G. Purdom, L. Nelsen, D. A. Hume, M. Rehli, and M. C. Ostrowski. 2002. The microphthalmia transcription factor and the related helix-loop-helix zipper factors TFE-3 and TFE-C collaborate to activate the tartrate-resistant acid phosphatase promoter. *J. Leukocyte Biol.* 71: 304–310.
- Yasumoto, K., and S. Shibahara. 1997. Molecular cloning of cDNA encoding a human TFEC isoform, a newly identified transcriptional regulator. *Biochim. Biophys. Acta* 1353: 23–31.
- Steingrimsdottir, E., L. Tessarollo, B. Pathak, L. Hou, H. Arnheiter, N. G. Copeland, and N. A. Jenkins. 2002. Mitf and Tfe3, two members of the Mitf-Tfe family of bHLH-Zip transcription factors, have important but functionally redundant roles in osteoclast development. *Proc. Natl. Acad. Sci. USA* 99: 4477–4482.
- Luchin, A., S. Suchting, T. Merson, T. J. Rosol, D. A. Hume, A. I. Cassady, and M. C. Ostrowski. 2001. Genetic and physical interactions between Microphthalmia transcription factor and PU.1 are necessary for osteoclast gene expression and differentiation. *J. Biol. Chem.* 276: 36703–36710.
- Hume, D. A., I. L. Ross, S. R. Himes, R. T. Sasmono, C. A. Wells, and T. Ravasi. 2002. The mononuclear phagocyte system revisited. *J. Leukocyte Biol.* 72: 621–627.
- Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23–35.
- Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
- Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol.* 163: 3920–3927.
- Welch, J. S., L. Escoubet-Lozach, D. B. Sykes, K. Liddiard, D. R. Greaves, and C. K. Glass. 2002. TH2 cytokines and allergic challenge induce Ym1 expression in macrophages by a STAT6-dependent mechanism. *J. Biol. Chem.* 277: 42821–42829.
- McKenzie, A. N. 2000. Regulation of T helper type 2 cell immunity by interleukin-4 and interleukin-13. *Pharmacol. Ther.* 88: 143–151.
- Herbert, D. R., C. Holscher, M. Mohrs, B. Arendse, A. Schwegmann, M. Radwanska, M. Leeto, R. Kirsch, P. Hall, H. Mossman, et al. 2004. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20: 623–635.
- Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176: 287–292.
- Willment, J. A., H. H. Lin, D. M. Reid, P. R. Taylor, D. L. Williams, S. Y. Wong, S. Gordon, and G. D. Brown. 2003. Dectin-1 expression and function are enhanced on alternatively activated and GM-CSF-treated macrophages and are negatively regulated by IL-10, dexamethasone, and lipopolysaccharide. *J. Immunol.* 171: 4569–4573.
- de Waal, M. R., C. G. Figdor, R. Huijbens, S. Mohan-Peterson, B. Bennett, J. Cuipepper, W. Dang, G. Zurawski, and J. E. de Vries. 1993. Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes: comparison with IL-4 and modulation by IFN- γ or IL-10. *J. Immunol.* 151: 6370–6381.
- Bonecchi, R., S. Sozzani, J. T. Stine, W. Luini, G. D'Amico, P. Allavena, D. Chantry, and A. Mantovani. 1998. Divergent effects of interleukin-4 and interferon- γ on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* 92: 2668–2671.
- Imai, T., M. Nagira, S. Takagi, M. Kakizaki, M. Nishimura, J. Wang, P. W. Gray, K. Matsushima, and O. Yoshie. 1999. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* 11: 81–88.
- Munder, M., K. Eichmann, J. M. Moran, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol.* 163: 3771–3777.
- Fernandez, P. B., F. C. Lucibello, M. Zuzarte, P. Lutjens, R. Muller, and K. Havemann. 2001. Dendritic cells derived from peripheral monocytes express endothelial markers and in the presence of angiogenic growth factors differentiate into endothelial-like cells. *Eur. J. Cell Biol.* 80: 99–110.

26. St. Pierre, B., G. Flock, E. Zacksenhaus, and S. E. Egan. 2002. Stra13 homodimers repress transcription through class B E-box elements. *J. Biol. Chem.* 277: 46544–46551.
27. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23: 549–555.
28. Lieschke, G. J., D. Grail, G. Hodgson, D. Metcalf, E. Stanley, C. Cheers, K. J. Fowler, S. Basu, Y. F. Zhan, and A. R. Dunn. 1994. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* 84: 1737–1746.
29. Shieh, J. H., R. H. Peterson, and M. A. Moore. 1991. Modulation of granulocyte colony-stimulating factor receptors on murine peritoneal exudate macrophages by tumor necrosis factor- α . *J. Immunol.* 146: 2648–2653.
30. Boneberg, E. M., L. Hareng, F. Gantner, A. Wendel, and T. Hartung. 2000. Human monocytes express functional receptors for granulocyte colony-stimulating factor that mediate suppression of monokines and interferon- γ . *Blood* 95: 270–276.
31. Ohsaka, A., K. Saionji, T. Kuwaki, T. Takeshima, and J. Igari. 1995. Granulocyte colony-stimulating factor administration modulates the surface expression of effector cell molecules on human monocytes. *Br. J. Haematol.* 89: 465–472.
32. Zhan, Y., S. Basu, G. J. Lieschke, D. Grail, A. R. Dunn, and C. Cheers. 1999. Functional deficiencies of peritoneal cells from gene-targeted mice lacking G-CSF or GM-CSF. *J. Leukocyte Biol.* 65: 256–264.
33. Sloand, E. M., S. Kim, J. P. Maciejewski, F. Van Rhee, A. Chaudhuri, J. Barrett, and N. S. Young. 2000. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. *Blood* 95: 2269–2274.
34. Anderson, K. L., K. A. Smith, F. Pio, B. E. Torbett, and R. A. Maki. 1998. Neutrophils deficient in PU.1 do not terminally differentiate or become functionally competent. *Blood* 92: 1576–1585.
35. DeKoter, R. P., J. C. Walsh, and H. Singh. 1998. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *EMBO J.* 17: 4456–4468.